

# Influenza virus-inhibitory effects of a series of germanium- and silicon-centred polyoxometalates

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## Summary

A series of germanium- or silicon-centred heteropolytungstates (polyoxometalates) with the Barrel, Keggin or double Keggin structure were evaluated *in vitro* for their effects against influenza A (IV-A) and B (IV-B) viruses. Their 50% effective concentrations ( $EC_{50}$ ) against recent isolates of IV-A (H1N1) and IV-B ranged from 0.1 to 7.8  $\mu$ M; against IV-A (H3N2), the  $EC_{50}$  concentrations were often 10-fold higher. Recent clinical isolates of IV-A were generally more susceptible to these antiviral effects than older, laboratory-adapted strains. These experiments used inhibition of viral CPE in MDCK cells as determined microscopically and by Neutral Red (NR) uptake. Virus yield reduction studies indicated the 90% effective concentrations ( $EC_{90}$ ) ranged from 0.2 to 32  $\mu$ M against these viruses. Cytotoxic or cell inhibitory concentrations ( $CC_{50}$ ), determined by NR uptake and total cell count, ranged from 38 to 189  $\mu$ M, indicating high selective indices for some of these compounds. Altering time of addition of an active compound relative to infecting cells with IV-A (H1N1) showed greatest efficacy when given early in viral replication. Five of the most active polyoxometalates were evaluated against IV-B infections in mice using intraperitoneal treatment beginning 4 h prior to virus exposure. Two of the compounds, one with the Barrel structure and the other with

a double Keggin structure, were particularly inhibitory, preventing deaths, reducing arterial oxygen decline and lowering lung consolidation. Lung virus titres were reduced by a maximum of 0.7  $\log_{10}$ . Therapy initiated 8 h post-virus exposure was not effective against this *in vivo* infection.

**Keywords:** Influenza virus; antiviral; animal model; *in vitro* assay; polyoxometalate; heteropolytungstate.

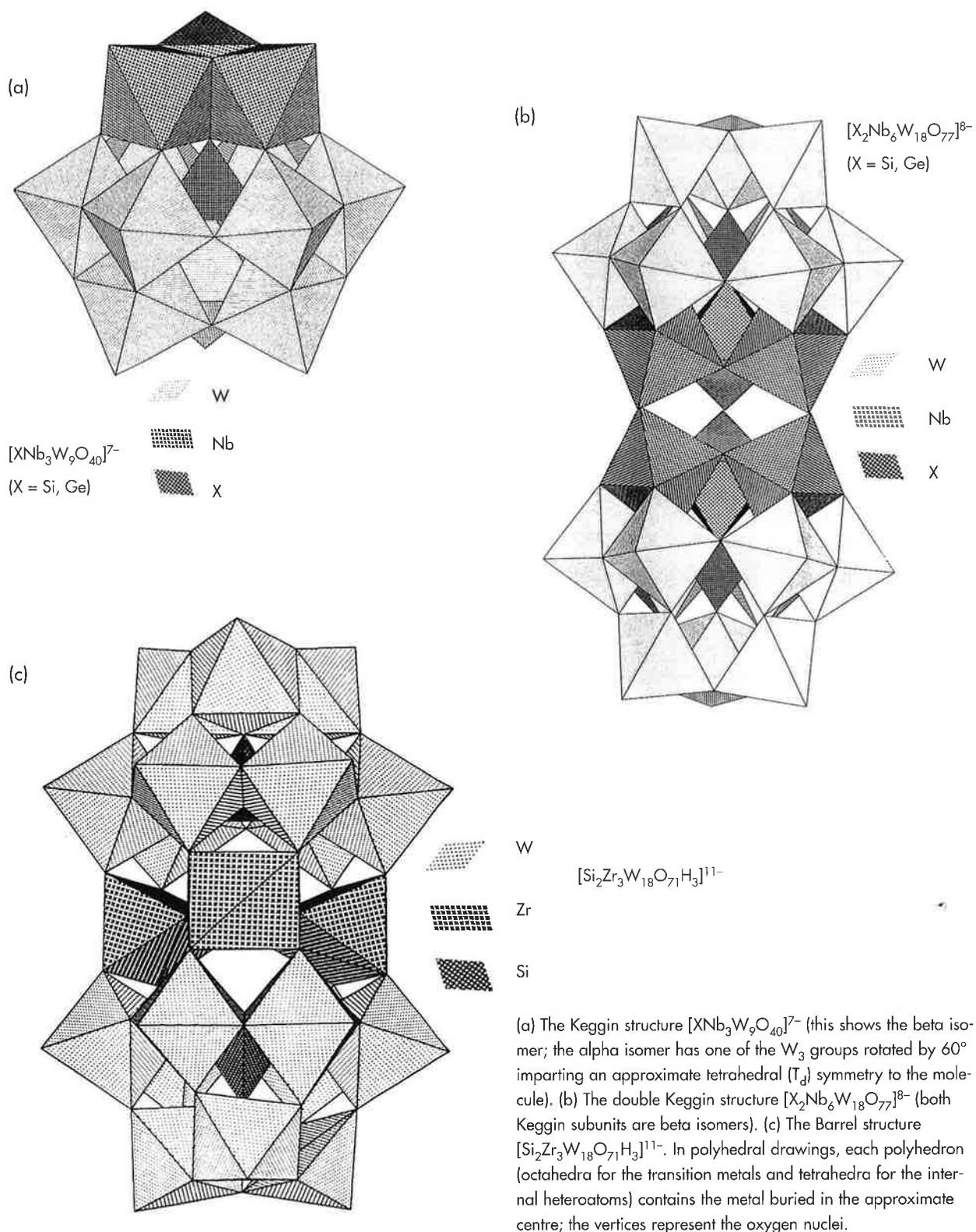
## Introduction

The novel early transition metal oxygen anion clusters known as polyoxometalates (POM) have been described in recent years by numerous investigators as having a spectrum of antiviral activities, primarily on enveloped viruses. Several studies have indicated that certain POM are significantly inhibitory to human immunodeficiency virus (HIV) *in vitro* (Hill *et al.*, 1990, 1993; Yamamoto *et al.*, 1992; Inouye *et al.*, 1993; Blasecki, 1994; Judd *et al.*, 1994; Kim *et al.*, 1994; Swart & Meijer, 1994; Moore *et al.*, 1995; Shigeta *et al.*, 1995). Herpesviruses have been reported to be inhibited by some of these compounds both *in vitro* (Fukuma *et al.*, 1991; Yamamoto *et al.*, 1992; Ikeda *et al.*, 1993) and *in vivo* (Ikeda *et al.*, 1994). Other viruses inhibited include toga-, rhabdo-, arena-, paramyxo- and murine retroviruses (Yamamoto *et al.*, 1992; Ikeda *et al.*, 1993; Shigeta *et al.*, 1995). Of particular pertinence to the present study has been the reported activity of POM against influenza A and B viruses (Ikeda *et al.*, 1993; Shigeta *et al.*, 1995), the inhibitory materials being polyoxotungstates with the 'Keggin' or 'Keggin sandwich' structure, with a number of these materials containing silicon.

A new series of silicon- or germanium-centred POM of the 'Barrel', 'Keggin' or 'Double Keggin' structure was synthesized and examined for their influenza A (H1N1, H3N2) and B virus-inhibitory effects *in vitro* with follow-up experiments run against influenza B virus infections in mice. The molecular formulae of these compounds are given in Table 1 and Fig. 1 shows their general structures. This report describes the antiviral activity of these materials.

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**Figure 1.** Polyhedral drawings of the three structural families of drugs evaluated in this study.



## Materials and Experimental Procedures

### Viruses

Influenza viruses used were A/NWS/33 (H1N1), A/Texas/36/91 (H1N1), A/Beijing/32/92 (H3N2), A/Port Chalmers/1/73 (H3N2), B/Panama/45/90 and B/Hong Kong/5/72. The NWS virus was provided by KW Cochran (University of Michigan, Ann Arbor, Mich., USA); the Port Chalmers and Hong Kong viruses were obtained from the ATCC (Rockville, Md., USA); the Texas, Beijing and Panama viruses were provided by H Regnery of the Influenza Branch, CDC (Atlanta, Ga., USA). Each virus used was from pools prepared in Madin-Darby canine kidney (MDCK) cells; each pool had been ampuled and stored at  $-80^{\circ}\text{C}$ . Each was pre-titrated in MDCK cells. The B/Hong Kong/5/72 virus, also used in animals experiments, was titrated in mice prior to use in this study.

### Cells and media

The MDCK cells used were obtained from the ATCC. They were maintained in antibiotic-free MEM with non-essential amino acids (Gibco) containing 5% foetal bovine serum (HyClone Laboratories) and 0.1%  $\text{NaHCO}_3$ . The cells were determined to be free of mycoplasma. Test medium used in antiviral experiments was MEM with 0.18%  $\text{NaHCO}_3$ , 10  $\text{U mL}^{-1}$  trypsin, 1  $\mu\text{g mL}^{-1}$  EDTA and 50  $\mu\text{g mL}^{-1}$  gentamicin.

### Compounds

The POM used in this study are shown in Table 1. Four of these compounds had the double Keggin and one an alpha Keggin structure shown in a previous publication (Hill *et al.*, 1995). Compounds JM-2919, JM-2922 and JM-2923 were synthesized according to published procedures. For JM-2919, the method reported by Finke *et al.* (1989) was used. Synthesis of JM-2922 and JM-2923 was as reported by Finke & Droege (1984). Compounds JM-2921,

JM-2925, JM-2926 and JM-2927 were gifts of Johnson-Matthey Corporation (West Chester, Penn., USA). Ribavirin, used as a positive control, was provided by ICN Pharmaceuticals.

### Animals

Female specific pathogen-free BALB/c mice weighing 12–15 g were obtained from Simonsen Laboratories (Gilroy, Calif., USA). They were quarantined 24 h prior to use and fed standard mouse chow and tap water. Once infected, they were given drinking water containing 0.006% oxytetracycline (Pfizer; New York, N.Y., USA) to control possible secondary bacterial infections.

### In vitro antiviral experimental procedures

Three methods were used to assay *in vitro* antiviral activity; inhibition of viral CPE determined by visual (microscopic) examination; increase in Neutral Red (NR) dye uptake; and decrease in virus yield.

The CPE inhibition method was run as previously described (Sidwell & Huffman, 1971; Sidwell *et al.*, 1985); briefly, seven 0.5  $\log_{10}$  dilutions of each compound were added to 6 wells in 96-well flat-bottomed microtitre plates (Corning, N.Y., USA) containing the cell monolayer. Within 5 min, the virus at a concentration of 50 cell culture 50% infectious doses ( $\text{TCID}_{50}$ ) was added to four wells at each compound concentration. Sterile virus diluent was added to the remaining two wells which served as toxicity controls. The plate was sealed, incubated at  $37^{\circ}\text{C}$  and CPE read at 72 to 96 h, when untreated infected controls developed near maximum (3–4+) CPE.

The NR dye uptake test was run to validate the CPE inhibition seen in the initial test. After the CPE was read, NR was added to the plate used in the CPE inhibition test according to the method of McManus (1976), adapted to antiviral tests as we have recently described (Sidwell *et al.*, 1995). The degree of colour intensity was read on a computerized EL-309 microplate autoreader (Bio-Tek

**Table 1.** Formulae and class of POM studied.

Number	Mol. Wt.	Formula	Class
JM-2919	5597	$(\text{Me}_3\text{NH})_{10}[\text{H}][\text{Si}_2\text{Zr}_3\text{W}_{18}\text{O}_{71}\text{H}_3] \cdot 10 \text{H}_2\text{O}$	Barrel
JM-2921	3244	$\text{K}_7[\text{A}-\alpha\text{-GeNb}_3\text{W}_9\text{O}_{40}] \cdot 18\text{H}_2\text{O}$	Alpha Keggin
JM-2922	3235	$\text{K}_7[\text{A}-\beta\text{-SiNb}_3\text{W}_9\text{O}_{40}] \cdot 20\text{H}_2\text{O}$	Beta Keggin
JM-2923	5713	$(\text{Me}_3\text{NH})_8[\text{Si}_2\text{Nb}_6\text{W}_{18}\text{O}_{77}]$	Double Keggin
JM-2925	5802	$(\text{Me}_3\text{NH})_8[\text{Ge}_2\text{Nb}_6\text{W}_{18}\text{O}_{77}]$	Double Keggin
JM-2926	5843	$\text{K}_7[\text{H}][\text{A}-\alpha\text{-Ge}_2\text{Nb}_6\text{W}_{18}\text{O}_{77}] \cdot 18 \text{H}_2\text{O}$	Double Keggin
JM-2927	5468	$\text{K}_8[\text{A}-\beta\text{-Si}_2\text{Nb}_6\text{W}_{18}\text{O}_{77}]$	Double Keggin

Instruments). Antiviral activity for both CPE and the NR uptake test methods was expressed as the 50% effective concentration ( $EC_{50}$ ).

Compounds considered active by CPE inhibition and by NR dye uptake were further tested using reduction in virus yield. This test was run in a similar manner to the CPE inhibition assay, with the plate frozen and thawed and eluates from each set of wells assayed for virus titre by serial dilution onto MDCK cell monolayers. The virus was allowed to adsorb to the cells at 37°C; the inoculum was then removed, the cells rinsed and fresh medium added to each well before the cells were incubated to allow CPE to develop as an indication of the presence of infectious virus. A 90% effective concentration ( $EC_{90}$ ), which was the test drug concentration that inhibited virus yield by 1 log<sub>10</sub> was determined from these data. Ribavirin was included in each test as a known positive control. Stock solutions of POM were prepared in water (JM-2919, -2921, -2922 and -2927) at 20 mM or in an equal volume mixture of water and DMSO (JM-2923, -2925 and -2926) at 10 mM before dilution in MEM for testing. Stock solutions were stored at -20°C between tests. Ribavirin was prepared and diluted in MEM.

### Cytotoxicity determinations

Each antiviral test plate included two wells with uninfected cells at each test compound concentration, which were used for toxicity controls. Initial cytotoxicity was determined using microscopically visible cell anomalies as previously described (Sidwell *et al.*, 1985). This visually determined cytotoxicity was then confirmed by NR dye uptake as described above. A 50% cytotoxic concentration ( $CC_{50}$ ) was then determined. Compounds with significant antiviral activity were further studied for their cytotoxic effects by evaluating varying concentrations in cell growth medium. Twelve-well tissue culture plates (Corning) were seeded with cells sufficient to be 20% confluent in each well so the cells were rapidly dividing during a 72 h incubation at 37°C. Cells were allowed to attach to the plates during a 4 h incubation without compound. The seeding medium was then removed and compound dilutions in warmed cell growth medium were placed on the cells and the plates incubated at 37°C. Following incubation, the cells were washed, trypsin was added to remove them from the plate, and the total cell number determined using a Model F Coulter Counter (Coulter Electronics, Hialeah, Fla., USA). The  $CC_{50}$  was determined using the average of three separate counts on each of two separate wells at each drug dilution.

### In vivo antiviral experiment procedures

Mice were anaesthetized by intraperitoneal (i.p.) injection of 60 mg kg<sup>-1</sup> of sodium pentobarbital and infected intranasally with a 75% lethal dose ( $LD_{75}$ ) of influenza

B/Hong Kong/5/72 virus. Compounds JM-2919, JM-2921, JM-2923, JM-2925 and JM-2927 in doses of 2, 6 and 20 mg kg<sup>-1</sup> day<sup>-1</sup> were administered i.p. to the mice once daily for 5 days beginning 4 h pre-virus exposure. Ribavirin was administered i.p. in parallel as a positive control at a dose of 100 mg kg<sup>-1</sup> day<sup>-1</sup> twice daily for 5 days beginning 4 h pre-virus exposure. Ten infected animals in each treatment group and 20 infected, saline-treated controls were held for 21 days with deaths recorded daily. Arterial oxygen saturation ( $SO_2$ %) determined using a pulse oximeter as described previously (Sidwell *et al.*, 1992) was assayed on days 3 through 10 in these animals. Three additional infected, treated mice in each group were euthanized on days 2, 4, 6, 8 and 10 and their lungs assigned a lung score ranging from 0 (normal) to 4 (maximum plum coloration) and lung virus titre was determined by assay of dilutions of lung homogenates in MDCK cells as described previously (Sidwell *et al.*, 1985). Three un-infected mice treated with each drug dose and three normal controls were run in parallel as toxicity controls. Deaths and weight gain were determined for these animals. A later experiment was run as above using JM-2925 administered at 2, 6, 20 and 30 mg kg<sup>-1</sup> day<sup>-1</sup> beginning 8 h post-virus exposure. In this experiment, lung parameters were determined on day 6 only.

### Data analysis (in vitro experiments)

Selective indices (SI) were determined as  $CC_{50}/EC_{50}$  in the CPE and NR tests and  $CC_{50}/EC_{90}$  in the virus yield test, the latter  $CC_{50}$  being that determined in rapidly dividing cells. Correlation of CPE and NR data was done with the Pearson correlation coefficient (PCC) method using the computerized Minitab Release 10extra program (Minitab) on a Macintosh computer.

### Data analysis (in vivo experiments)

Increase in survivor numbers was evaluated using chi-squared analysis with Yates' correction. Mean day to death increases,  $SO_2$ % differences and virus titre reductions were analysed by Student's *t*-test. Lung score inhibition was evaluated by ranked sum analysis.

## Results

### Antiviral effect using CPE inhibition and NR uptake tests

Initial antiviral experiments used three recent clinical isolates of influenza virus selected because they were being targeted by current vaccines. These viruses included A/Texas/36/91 (Texas, H1N1), A/Beijing/32/92 (Beijing, H3N2) and B/Panama/45/90 (Panama). When significant activity, defined as an SI of 10 or greater, was seen using these viruses, follow-up studies were run using other, more

**Table 2.** *In vitro* influenza virus-inhibitory effects of a series of germanium- and silicon-centred POM.

Virus	Compound															
	JM-2919		JM-2921		JM-2922		JM-2923		JM-2925		JM-2926		JM-2927		Ribavirin	
	EC <sub>50</sub> <sup>a</sup>	SI	EC <sub>50</sub>	SI	EC <sub>50</sub>	SI	EC <sub>50</sub>	SI	EC <sub>50</sub>	SI	EC <sub>50</sub>	SI	EC <sub>50</sub>	SI	EC <sub>50</sub>	SI
A/Texas	0.2	345	0.25	>400	0.95	>105	0.1	>960	0.15	>667	0.15	613	0.2	355	8.6	72
A/NWS	6.5	11	6.6	>15	17	>6	5.2	>18	5.6	>18	6.6	14	6.2	11	19.2	32
A/Beijing	5.2	13	15.5	>6.4	48	>2	3.0	>32	5.0	>20	5.6	16	11.5	6.2	7.6	81
A/Port Chalmers	7.2	10	5.2	>19	26	>3.8	8.6	>11	13.5	>7.4	25.5	3.6	36	2.0	79.9	7.8
B/Panama	0.25	276	5.0	>20	6.8	>15	2.6	>37	0.4	>250	0.4	230	0.15	473	11.5	54
B/Hong Kong	6.6	10	0.6	>167	13	>7.7	0.13	>738	1.2	>83	1.8	51	1.6	44	5.8	107
Cytotoxicity																
CC <sub>50</sub> <sup>b</sup> (μM)	69		>100		>100		>96		>100		92		71		620	

<sup>a</sup> Mean (μM) determined by CPE and NR uptake.<sup>b</sup> Mean (μM) determined by NR uptake.

laboratory-adapted related viruses.

Table 2 summarizes the inhibitory effects of these compounds on four strains of influenza A virus and two strains of influenza B virus as determined by CPE inhibition observed microscopically and by NR dye uptake. The CPE inhibition and the NR uptake tests gave quite similar EC<sub>50</sub> results, although the values obtained from the NR uptake test tended to be slightly higher. The PCC value for the data obtained with the two H1N1 viruses was 0.948; a perfect correlation would yield a value of 1.0, so this value indicates an almost 95% correlation of the data. The PCC value for data obtained with the H3N2 viruses was 0.914 and the value obtained with the influenza B viruses was 0.975. Because of the high correlation, the EC<sub>50</sub> values obtained by CPE and by NR uptake were combined to give the mean values shown in Table 2. A precipitate was usually encountered at the higher concentrations of these POM when incubated with the cell culture medium in this study. These precipitates tended to obscure reading of either cytotoxicity or CPE by microscope, but the cells were still sufficiently viable to take up the NR dye at these high dosages.

The recent clinical isolate Texas strain of the H1N1 virus was more sensitive than the laboratory-adapted (NWS) virus, with all of the POM studied being 18 to 52 times more potent against the former virus. The H3N2 influenza viruses were generally less sensitive to these POM than the H1N1 viruses. This was seen using both a recent clinical isolate (Beijing) and a laboratory-adapted virus (Port Chalmers). The Beijing isolate was usually more sensitive than the Port Chalmers virus, both to the POM and to ribavirin. Compounds JM-2919, JM-2923 and JM-2925 were considered most efficacious in this

study with these H3N2 viruses. JM-2926 was also significantly inhibitory to the Beijing virus, but was less effective against the Port Chalmers strain.

Significant inhibition of both Panama and Hong Kong strains of influenza B virus was noted using these POM. The potency of some compounds was greater against the Panama strain than against the Hong Kong strain, but for other compounds the potency was greater against the Hong Kong strain. SI values exceeding 100 were noted against one or the other virus with all compounds but JM-2922. The compound was also the least potent against the other viruses.

#### *Inhibitory effects on virus yield*

Virus yield reduction experiments were carried out with the viruses used in initial testing, if significant inhibition was seen with these viruses in the initial testing. The EC<sub>90</sub> data, together with the CC<sub>50</sub> determinations made using rapidly metabolizing cells are summarized in Table 3. Comparing SI values, these results generally confirm the positive activities seen by CPE inhibition and NR dye uptake against the H1N1 and B viruses. Only JM-2925 was evaluated using virus yield inhibition with the H3N2 virus, and significant efficacy was seen. The CC<sub>50</sub> determined by cell count in proliferating cells compared well with those obtained in the confluent cells.

#### *Influence of time of addition of JM-2919 on antiviral activity*

As an initial step in determining the mechanism of action of these compounds, a timing experiment was run in which JM-2919 was added to cells before, during or after the cells were exposed to the Texas (H1N1) strain of

**Table 3.** Virus yield-inhibitory effects of a series of germanium- and silicon-centred POM.

Virus	Compound											
	JM-2919		JM-2921		JM-2922		JM-2923		JM-2925		JM-2926	
	EC <sub>90</sub> <sup>a</sup>	SI	EC <sub>90</sub>	SI	EC <sub>90</sub>	SI	EC <sub>90</sub>	SI	EC <sub>90</sub>	SI	EC <sub>90</sub>	SI
A/Texas	0.2	945	1.2	110	9.3	16	0.2	205	1.1	58	0.9	141
A/Beijing	ND <sup>b</sup>		ND		ND		ND		0.5	128	ND	
B/Panama	32	6	9.3	14	3.3	44	0.04	103	1.3	49	0.7	181
<i>Cytotoxicity</i>												
CC <sub>50</sub> <sup>c</sup> (µM)	189		132		145		41		64		127	

<sup>a</sup> µM.<sup>b</sup> Not done.<sup>c</sup> Determined by cell count using rapidly dividing MDCK cells.

influenza virus. The virus was added at a low m.o.i. (0.002 cell<sup>-1</sup>) to be able to show a wide range of activity. Antiviral activity was ascertained using both CPE inhibition and NR uptake methods, with the test compound added (i) 24 h pre-virus exposure and then removed and the cells fully washed before addition of virus; (ii) 24 h pre-virus, removed, and then added again at the same time as the virus and allowed to remain on the cells for the 72 h duration of the experiment; (iii) 1 h; (iv) 8 h; or (v) 24 h after virus exposure and allowed to incubate on the infected cells for the remainder of the 72 h duration of the study. Greatest efficacy was seen when this POM was added nearest to the time of virus exposure, when viral adsorption and penetration were occurring. Delaying addition for 1 h

(after viral absorption but during penetration phase) had a lesser effect. Additions at late stages in viral replication (8 or 24 h post-virus exposure) or pretreatment and removal before addition of virus were at least eight times less effective than when added at the same time as the virus. Two other POM, JM-2921 and JM-2925, were also evaluated in timing experiments with similar activity as described above for JM-2919 (data not shown).

#### *In vivo antiviral activity*

The results of an experiment using JM-2925 are summarized in Table 4. Owing to the extensive nature of the data in which mice were euthanized on multiple days during the experiment for assay of lung parameters, only the day

**Table 4.** Effect of intraperitoneal treatment with JM-2925 on an influenza B virus infection in mice.

Compound	Dosage <sup>a</sup> (mg kg <sup>-1</sup> day <sup>-1</sup> )	Toxicity controls		Infected, treated mice				
		Survival/total	Mean wt change (g) <sup>b</sup>	Survival/total	Mean days to death <sup>c</sup> ±SD	Day 10 SaO <sub>2</sub> % (mean±SD)	Day 10 lung score (mean±SD)	Day 2 lung virus titre (mean log <sub>10</sub> TCID <sub>50</sub> g <sup>-1</sup> ±SD)
JM-2925	20	3/3	1.2	9/10**	7.0±0.0	83.8±8.1**	2.5±0.6*	5.0±0.4
	6	3/3	1.7	1/10	9.4±2.6	76.0±9.1	2.5±0.0*	4.6±0.1
	2	3/3	1.3	7/10*	7.7±0.3	79.5±7.8	2.8±0.3	4.5±0.3
Ribavirin	100	3/3	-0.6	8/10**	7.5±0.3	82.8±7.8*	0.7±1.2**	4.8±0.4
Saline	-	-	-	5/20	9.3±1.9	73.1±5.9	3.3±0.3	5.2±0.4
Normal controls	-	3/3	0.8	-	-	85.2±1.3	0.0±0.0	0.0±0.0

<sup>a</sup> Once daily for 5 days beginning 4 h pre-virus exposure.<sup>b</sup> Difference between initial weight at start of treatment and weight 18 h following final treatment.<sup>c</sup> Mice dying on or before day 21.\*  $P < 0.05$ ; \*\*  $P < 0.01$ .

10 SaO<sub>2</sub>% and lung consolidation data and day 2 lung virus titre data are shown. Maximum effects were seen on these days. Treatment with the POM significantly prevented deaths in the mice, reduced the usual decline in SaO<sub>2</sub>% and moderately inhibited lung consolidation. Lung virus titres were slightly reduced, the maximum titre reduction being 0.7 log<sub>10</sub>. The prevention of deaths was not fully dose-responsive, with significant numbers of survivors seen at the high and low drug dose but not the middle dose.

The compound was well tolerated by the mice, with all toxicity controls surviving and gaining weight. In a later experiment the maximum dose was increased to 30 mg kg<sup>-1</sup> day<sup>-1</sup>, and again all toxicity control mice survived but gained only 0.1 g of weight, suggesting the maximum tolerated dose was being approached.

A second similar experiment using JM-2925 against an influenza B virus infection in mice with treatment beginning 8 h after virus exposure did not indicate any antiviral activity using this compound; no increase in survivor number or mean day to death, prevention of SaO<sub>2</sub> decline, or inhibition of lung consolidation or virus titre was seen (data not shown).

Five of these POM were evaluated in a similar manner to the initial experiment described above with JM-2925. Two of the compounds, JM-2921 and JM-2923, were run in parallel with JM-2925 in the same experiment. The data using all five compounds is summarized in Table 5, with only the maximum protective effect using each disease parameter shown. JM-2925 was considered most efficacious, with the order of activity for the compounds being JM-2925>JM-2919>JM-2921>JM-2926>JM-2923.

## Discussion

These experiments indicate that this series of POM has strong inhibitory effects against influenza A (H1N1) and B viruses, with less effect against the H3N2 viruses. These results extend the findings of Shigeta *et al.* (1995) in which a series of POM, some of which also contained silicon but different from those used in the present experiments, were found inhibitory to influenza A (H3N2) (A/Ishigawa/7/82) and B (B/Singapore/222/79) viruses. The EC<sub>50</sub> values determined in the present study, albeit with different strains of virus, were significantly higher with this series of POM than with those reported by Shigeta *et al.* (1995). Ikeda *et al.* (1993) also evaluated a series of polyoxosilicotungstates for efficacy against orthomyxoviruses, utilizing the same viruses as used by Shigeta *et al.* (1995). They found SI values in the 15 to >50 range against influenza A virus, but generally a weaker activity against influenza B virus. To the best of our knowledge, no studies addressing the antiviral properties of germanium-centred POM have been reported.

As reviewed earlier, POM have been reported to have antiviral activity against a variety of viruses; such data suggest a non-selective mechanism of action for these materials. We have also studied the compounds described in this report for activity against other enveloped viruses, including measles virus, parainfluenza virus type 3 and respiratory syncytial virus. A striking inhibitory effect was seen versus several strains of respiratory syncytial virus (DL Barnard, JH Huffman, RW Sidwell, A Morrison, MJ Otto, CL Hill & RF Schinazi, unpublished results), but surprisingly the measles and parainfluenza viruses were not inhibited or were affected only at high concentrations

**Table 5.** Protective effects seen in influenza B virus-infected mice treated with germanium- or silicon-centred POM.

Compound	Dose range (mg kg <sup>-1</sup> day <sup>-1</sup> ) <sup>a</sup>	Maximum values				
		Survivor increase <sup>b</sup> (%)	Mean day to death increase (days)	Inhibition of SaO <sub>2</sub> decline (%)	Lung score decrease (%)	Lung virus titre reduction (log <sub>10</sub> TCID <sub>50</sub> g <sup>-1</sup> )
JM-2919	2–20	52**	1.7	13**	20	0.7
JM-2921	2–20	5	2.6	15**	24*	0.5
JM-2923	2–20	0	1.4	2	3	0.4
JM-2925	2–20	65**	0.1	15**	24*	0.7
JM-2926	2–20	25	2.5	10	20	0.4

<sup>a</sup> I.p. treatment once daily for 5 days beginning 4 h pre-virus exposure.

<sup>b</sup> LD<sub>75</sub> virus challenge used.

\* *P*<0.05; \*\* *P*<0.01 compared to values in concomitantly run placebo controls.

of the POM. The non-enveloped adenovirus was also not inhibited. Such data suggest these materials to be more selective than expected.

Studies using HIV-1 have shown that these large and negatively charged molecules are active at the cell surface (Inouye *et al.*, 1993; Judd *et al.*, 1994), although certain smaller-sized POM have been shown to readily penetrate cell membranes (Hill *et al.*, 1995). The latter observation suggests possible intracellular roles for the antiviral activity of these materials. RF Schinazi *et al.* (Presentation at the VI International Antiviral Symposium, Nice, France, 1994) have reported some of these materials selectively inhibit HIV-1 protease and Yamamoto *et al.* (1992) showed them to inhibit reverse transcriptase activity associated with HIV.

The various antiviral assay methods employed in the present study all appear to give reasonably comparable viral inhibition data as determined by PCC analysis; the presence of a precipitate at higher concentrations sometimes masked visual examination of cells for cytotoxic effects, but NR uptake appeared to take place in the cells, indicating they were still viable. The virus yield data also correlated well with the results obtained with CPE inhibition and NR uptake.

The m.o.i. used in the virus yield experiments was 0.002 cell<sup>-1</sup>; at this low m.o.i., one might expect a strong inhibition of virus with the POM, based on the results of experiments by Shigeta *et al.* (1995) in which treatment with POM of cells infected with high m.o.i. of virus did not significantly inhibit the infection, but in the presence of low m.o.i. good inhibition of virus yield occurred. This low m.o.i. viral challenge may also account for the moderate viral inhibition seen when the compound was added after virus exposure.

The animal studies reported indicate the *in vitro* influenza virus inhibition activity of these POM can also be seen *in vivo*, although efficacy was exhibited only when therapy began near the time of virus challenge, correlating with the *in vitro* timing studies. The strong efficacy seen with the high and the low, but not the middle dose, of JM-2925 suggests a possible immunomodulatory effect of these materials in addition to the more direct antiviral effect already discussed. Certain synthetic polymers, including polycarboxylates such as MVE-2 (1:2 divinyl ether: maleic anhydride cyclic copolymer) are known to have a spectrum of immunomodulatory effects, including macrophage activation, which has led to protection of mice from influenza virus (for review see Carrano *et al.*, 1984). Studies are underway in our laboratory to determine the immunomodulatory effects of these POM.

It is difficult to determine a definitive structure-activity relationship with the compounds used in this study, since essentially all exerted significant antiviral activity, although the Barrel and double Keggin structure POM

were most efficacious *in vivo*. It is apparent that POM that are either germanium- or silicon-centred and contain the Barrel, Keggin or particularly the double Keggin structure have a potent ortho- and paramyxovirus-inhibitory effect. Silicon-centred POM with a Keggin or double Keggin structure have previously been reported to have influenza virus-inhibitory effects (Ikeda *et al.*, 1993; Shigeta *et al.*, 1995). The present study indicates the germanium-centred molecules also have similar antiviral activity.

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